

This Page Is Inserted by IFW Operations  
and is not a part of the Official Record

## **BEST AVAILABLE IMAGES**

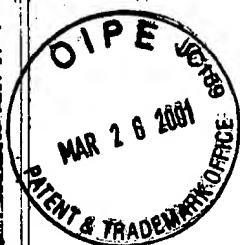
Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning documents *will not* correct images,  
please do not report the images to the  
Image Problem Mailbox.**



## Selective opening of the blood-tumor barrier by intracarotid infusion of leukotriene C<sub>4</sub>

KEITH L. BLACK, M.D., WESLEY A. KING, M.D., AND KIYONOBU IKEZAKI, M.D.

*The Brain Research Institute, The Jonsson Cancer Center, and the Division of Neurosurgery, University of California Medical Center, Los Angeles, California*

✓ Intracarotid infusions of leukotriene C<sub>4</sub> (LTC<sub>4</sub>) were used to selectively open the blood-tumor barrier in rats with RG-2 gliomas. Blood-brain and blood-tumor permeability was determined by quantitative autoradiography using <sup>14</sup>C aminoisobutyric acid. Leukotriene C<sub>4</sub> (4 μg total dose) infused into the carotid artery ipsilateral to the tumor increased twofold the unidirectional transfer constant for permeability within the tumor while no effect on permeability was seen in normal brain. No gamma glutamyl transpeptidase (γ-GTP) activity was seen in tumor capillaries in contrast to high γ-GTP in normal brain capillaries. These findings suggest that normal brain capillaries may resist the vasogenic effects of LTC<sub>4</sub>, while LTC<sub>4</sub> will increase permeability in tumor capillaries. This could relate to the ability of γ-GTP to act as an enzymatic barrier and inactivate leukotrienes in normal brain capillaries. Intracarotid LTC<sub>4</sub> infusion may be a useful tool to selectively open the blood-tumor barrier for delivery of antineoplastic compounds.

**KEY WORDS** • brain neoplasm • blood-brain barrier • glioma • leukotriene • gamma glutamyl transpeptidase • rat

CAPILLARIES within primary brain tumors contain a blood-tumor barrier which impairs delivery of many antitumor moieties to tumor cells within the brain.<sup>8,12</sup> This inability to deliver antineoplastic compounds across the blood-tumor barrier has lead some workers to increase their focus on strategies to open this barrier.<sup>10,12,14,16,20</sup> One approach has utilized intracarotid infusions of mannitol to osmotically disrupt the tight junctions of brain endothelial cells in patients with brain tumors.<sup>12,14,15,20</sup> Neuwelt, *et al.*,<sup>13</sup> have shown that osmotic barrier disruption can non-specifically increase the delivery of chemotherapeutic compounds and monoclonal antibodies to the brain. Osmotic disruption of the blood-brain barrier (BBB), however, results in only a modest increase in drug levels within the actual tumor compared to the large increase in drug levels in normal brain.<sup>11</sup> In effect, therefore, this technique increases the exposure of the normal brain parenchyma to the deleterious effects of the chemotherapeutic agents,<sup>20</sup> while suboptimal drug levels may actually reach the tumor cells. In this paper, we describe an experimental method that circumvents this problem. This technique selectively opens the barrier within the tumor but leaves the BBB intact.

In previous studies, we demonstrated a significant

correlation between tissue levels of leukotriene C<sub>4</sub> (LTC<sub>4</sub>) measured in brain tumors and the amount of vasogenic edema surrounding these tumors in man.<sup>5</sup> This, combined with our observation that brain capillaries have high affinity binding sites for LTC<sub>4</sub>,<sup>2</sup> led us to speculate that LTC<sub>4</sub> might modulate capillary permeability in brain tumors.<sup>3</sup> Here we report on the ability of intracarotid infusions of LTC<sub>4</sub> to selectively open the tumor barrier in an experimental glial tumor, and we discuss both the biological and clinical implications of this observation.

### Materials and Methods

Leukotriene C<sub>4</sub>\* was obtained at a concentration of 0.1 mg/ml in a 1:1 phosphate-buffered saline (PBS)-ethanol solution. Aliquots of LTC<sub>4</sub> were diluted to experimental concentrations in PBS and the final ethanol concentration was adjusted to 2.5%. Vials containing LTC<sub>4</sub> were sealed under nitrogen and stored at -80°C. Radiolabeled LTC<sub>4</sub> (14,15-<sup>3</sup>H(N)-LTC<sub>4</sub>) (38.4 Ci/mmol) and alpha-(1-<sup>14</sup>C)-aminoisobutyric acid

\* LTC<sub>4</sub> obtained from Cayman Chemicals, Ann Arbor, Michigan.

(AIB) (50.0 mCi/mmol) were used for quantitative study.<sup>†</sup>

The RG-2 glioma cell line was maintained in a monolayer culture in Dulbecco's minimum essential medium with 10% calf serum. Female Wistar rats, each weighing 100 to 150 gm. were anesthetized with intramuscular ketamine (50 mg/kg) and xylazine (0.8 mg/kg). Glial tumors were implanted into the left hemisphere by intracerebral injections of  $5 \times 10^4$  RG-2 glioma cells in 10  $\mu$ l of serum-free F10 medium. Thirteen days after tumor implantation, the rats were again anesthetized and a polyethylene (PE-10) catheter was inserted retrograde through the external carotid artery to the common carotid artery bifurcation ipsilateral to the tumor. The external carotid artery was then ligated. Both femoral arteries and one femoral vein were also cannulated.

Body temperature was maintained at 37°C and arterial blood gas levels, blood pressure, and hematocrit were monitored. Animals with abnormal physiological parameters were eliminated from this study. Leukotriene C<sub>4</sub> (0.8 ml) at a concentration of 5 µg/ml or 0.8 ml of vehicle (2.5% ethanol in PBS, pH 7.1) was infused into the left carotid artery by means of a constant infusion pump at a rate of 53.3 µl/min for 15 minutes. Five minutes after the start of the intracarotid infusion, 100 µCi/kg of <sup>14</sup>C-AIB was injected as an intravenous bolus. A peristaltic withdrawal pump was used to withdraw femoral arterial blood at a constant rate of 0.083 ml/min immediately after injection of <sup>14</sup>C-AIB for determination of serum radioactivity. Fifteen minutes after the start of intracarotid infusions, the animals were killed by decapitation and the brains were rapidly removed and frozen.

The frozen brains were mounted onto pedestals with M-1 embedding matrix, and 20  $\mu$ m coronal sections were cut with a cryotome. The sections were thaw-mounted onto cover slips, and autoradiograms were generated by coexposing the sections on Kodak XAR-5 film with tissue-calibrated  $^{14}\text{C}$  standards $\S$  for 2 weeks. After exposure, the sections were stained with thionine for correlation of areas of histologically verified tumor autoradiograms. Quantitative analysis was performed using a computer-assisted digital image analyzer at the Laboratory of Neuro-Imaging, University of California School of Medicine, Los Angeles.<sup>19</sup> A unidirectional blood-to-brain transfer constant,  $K_1$ , was calculated using the method described by Blasberg, *et al.*,<sup>6</sup> and was expressed in  $\mu\text{l/gm}/\text{min}$ .

To determine specific binding of  $^3\text{H}$ -LTC<sub>4</sub> to rat brain tissue and RG-2 tumors *in vitro*, brains from rats with and without RG-2 tumors were removed, frozen, and cut in 20- $\mu\text{m}$  coronal sections using a cryotome. The sections were separated in an alternate fashion to obtain adjacent sections to subtract nonspecific from total binding images. Specific  $^3\text{H}$ -LTC<sub>4</sub> binding images were obtained by subtracting nonspecific binding images from total binding images pixel by pixel, using a digital image analyzer. The sections were placed in a desiccator and kept in a freezer overnight. The sections were washed twice in ice-cold binding buffer (50 mM Tris-HCl, 10 mM MgCl<sub>2</sub>, 5 mM L-serine, 10 mM sodium borate, pH 7.4) for 4 minutes and cool-air dried; they were then immersed into binding buffer containing 2.5 nM  $^3\text{H}$ -LTC<sub>4</sub> for 60 minutes at 0° to 4°C with or without 10  $\mu\text{M}$  LTC<sub>4</sub>, and were again washed in binding buffer and cool-air dried. Autoradiograms were generated by exposing the sections of  $^3\text{H}$  Hyperfilm for 2 weeks with tissue-calibrated  $^3\text{H}$  standards. §

Rat brains with gliomas were fixed in 70% ethyl alcohol and embedded in paraffin. Six-micron sections were cut, deparaffinized, acetone-fixed, and incubated at 37°C for 60 minutes in a gamma glutamyl transpeptidase ( $\gamma$ -GTP) reaction mixture of 0.5 mM  $\gamma$ -glutamyl-4-methoxy-2-naphthylamide, 15mM glycylglycine, and 0.05% fast blue BB in 25 mM phosphate buffer (pH 7.2) containing 0.25% dimethylsulfoxide. Sections were then washed with distilled water, rinsed with 0.9% saline for 2 minutes, and placed in 0.1 M CuSO<sub>4</sub> for 2 minutes. Counterstaining of sections with hematoxylin was performed for 1 minute.

The  $K_d$  values for the experiments were calculated by measuring regions of interest in three consecutive sections. Analysis of variance and Student's *t*-test were applied to the mean values from separate experiments.

Intracarotid infusion of LTC<sub>4</sub> resulted in a twofold increase in brain-tumor barrier permeability ( $K_i$ ) within tumors which were 3 mm in diameter or smaller, when compared to vehicle alone (mean  $\pm$  standard error of the mean for nine rats in each group:  $45.58 \pm 6.61$  vs.  $22.33 \pm 2.35$   $\mu\text{l/gm/min}$ ,  $p < 0.003$ ). There was no significant change in BBB permeability of the ipsilateral cortex  $2.97 \pm 1.37$  vs.  $2.39 \pm 1.09$   $\mu\text{l/gm/min}$ , contralateral cortex  $2.87 \pm 1.36$  vs.  $2.80 \pm 1.27$   $\mu\text{l/gm/min}$ , or contralateral corpus callosum ( $2.30 \pm 0.92$  vs.  $0.89 \pm 0.53$   $\mu\text{l/gm/min}$ ). A modest increase in permeability

Nuclear, Boston, Massachusetts.  
 $\delta^{13}\text{C}$  standards obtained from Amersham Corp., Arlington Heights, Illinois.

913

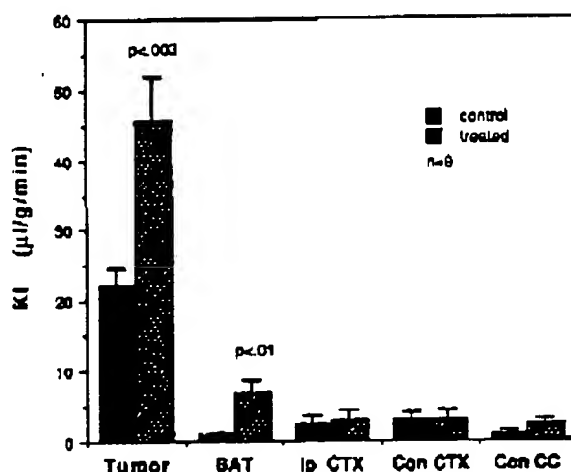
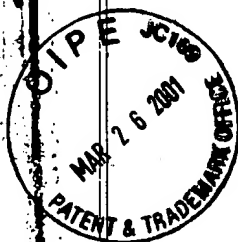


FIG. 1. Effect of intracarotid infusion of either leukotriene  $C_4$  (nine rats) or vehicle (nine rats) on blood-brain and blood-tumor barrier permeability. Values for the unidirectional transfer constant,  $K_t$ , are shown as the mean  $\pm$  standard error of the mean. BAT = brain adjacent to tumor; Ip CTX = ipsilateral cortex; Con CTX = contralateral cortex; Con CC = contralateral corpus callosum.

was seen in the basal ganglia immediately adjacent to the tumors ( $6.63 \pm 2.05$  vs.  $0.88 \pm 0.42$   $\mu\text{l/gm/min}$ ,  $p < 0.01$ ) although no tumor infiltration was seen in this area (Fig. 1). In tumors larger than 3 mm,  $K_t$  values ranged between 85 and 95  $\mu\text{l/gm/min}$  and were significantly not increased by  $\text{LTC}_4$  infusions.

The  $^3\text{H-LTC}_4$  binding experiments revealed some specific binding in normal brain ( $66.15 \pm 6.19$  fmol/mg tissue at 2.5 nM) but little specific binding in tumors ( $25.04 \pm 3.34$  fmol/mg tissue). There was no  $\gamma$ -GTP activity in tumor capillaries in contrast to normal brain (Fig. 2). Preliminary results suggested a slight decrease in  $\gamma$ -GTP levels in some areas of normal tissue surrounding tumors. The  $\text{LTC}_4$  infusions did not alter the physiological parameters.

#### Discussion

Leukotrienes are pharmacologically active compounds that promote vascular permeability.<sup>3,17,18,20</sup> This study demonstrates that the blood-tumor barrier in an experimental glial tumor can be selectively opened by intracarotid infusion of  $\text{LTC}_4$  without increasing permeability in the normal cortex or white matter. Clinically, intracarotid  $\text{LTC}_4$  infusions could therefore be an extremely useful tool to increase delivery of antitumor moieties within tumors without increasing the concentrations of potentially toxic compounds in normal brain. The mechanism(s) for the selective effect of  $\text{LTC}_4$  on capillary permeability in tumors compared to normal brain remains unclear. One possibility could reside in the ability of normal brain capillaries to resist the

K. L. Black, W. A. King, and K. Ikezaki

vasogenic effects of  $\text{LTC}_4$ . Leukotriene  $C_4$  will markedly increase permeability in a variety of (noncerebral) systemic capillary beds.<sup>17,18,21</sup> In prior studies, we have demonstrated that intraparenchymal injections into the brain of high doses of leukotrienes would increase BBB permeability.<sup>4</sup> However, lower physiological doses of  $\text{LTC}_4$  do not dramatically increase permeability in normal brain tissue (unpublished data). Brain capillaries, unlike systemic capillaries, are rich in  $\gamma$ -GTP,<sup>2</sup> an enzyme that metabolizes the peptidoleukotrienes  $\text{LTC}_4$  and  $\text{LTE}_4$  to  $\text{LTD}_4$  and  $\text{LTF}_4$ , respectively.<sup>1</sup> High levels of  $\gamma$ -GTP are not present in the capillaries of experimental tumors. Theoretically, at physiological concentrations,  $\text{LTC}_4$  could be inactivated by  $\gamma$ -GTP in normal capillaries, while tumor capillaries (which lack  $\gamma$ -GTP) are susceptible to the vasogenic effects of  $\text{LTC}_4$ .

In man, levels of  $\text{LTC}_4$  are significantly increased in tumors compared to normal tissue and there is a significant correlation between  $\text{LTC}_4$  levels and the amount of vasogenic edema surrounding these tumors.<sup>5</sup> If tumor capillaries are also more sensitive to the effects of  $\text{LTC}_4$ , as the present study suggests, this compound could play an important role in the formation of vasogenic edema surrounding brain tumors. Leukotrienes may also play a role in vasogenic brain edema formation in other pathological conditions where the resistance of normal brain capillaries to the effects of  $\text{LTC}_4$  may be impaired.

Telologically, the ability of normal brain capillaries to resist  $\text{LTC}_4$ -induced permeability and the inability of abnormal capillaries to do so could be particularly advantageous. Leukocytes, rich in the 5-lipoxygenase enzyme and a major source of leukotrienes, could effectively open capillaries in abnormal areas and enter the brain parenchyma where they are needed while normal areas could resist their effects.

Although high-affinity binding sites for  $\text{LTC}_4$  on isolated brain capillaries have been demonstrated,<sup>5</sup> it is not clear whether these binding sites are functional receptors for  $\text{LTC}_4$  or whether the receptor is located in a  $\gamma$ -GTP- $\text{LTC}_4$  receptor complex. *In vitro* binding experiments in this study showed a relative paucity of high-affinity binding sites within tumors. This observation raises several possibilities, including 1) that the binding site is a  $\gamma$ -GTP- $\text{LTC}_4$  receptor complex, which the tumors may lack, or 2) that the mechanism of increased permeability by  $\text{LTC}_4$  may not be receptor-mediated. These preliminary binding results do not, however, exclude the possibility of a functional  $\text{LTC}_4$  receptor on tumor capillaries since optimal binding conditions may not have been applied.

The increased permeability seen in the basal ganglia adjacent to tumor suggest that capillaries in these areas may also be abnormal. This could relate to tumor angiogenesis or other secondary effects induced by the tumor which may also make capillaries in these areas more susceptible to the effects of  $\text{LTC}_4$ . Of interest is the preliminary observation that  $\gamma$ -GTP levels appear

## Blood-tumor barrier opening by LTC<sub>4</sub> infusion

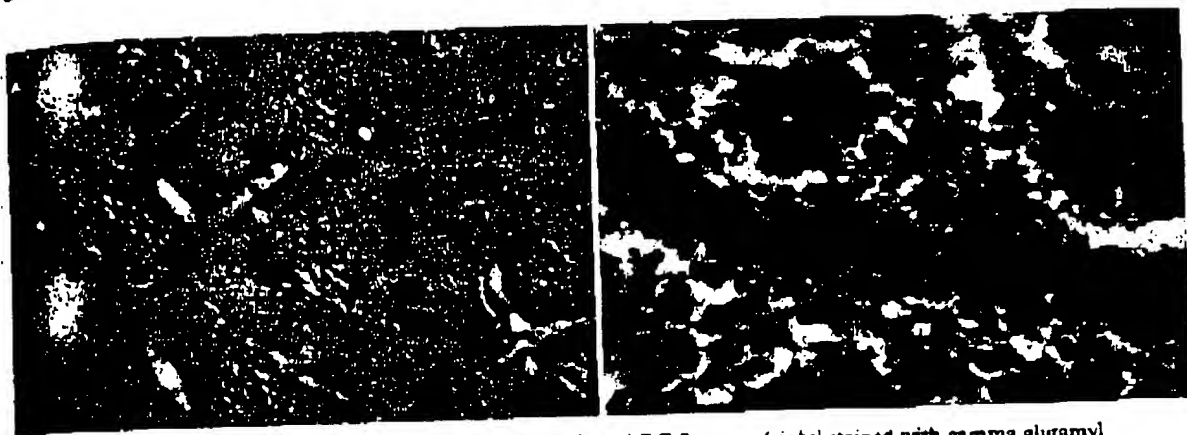


FIG. 2. Histological sections of normal brain (left) and RG-2 tumor (right) stained with gamma glutamyl transpeptidase ( $\gamma$ -GTP). Little staining is seen in the tumor compared to  $\gamma$ -GTP staining in capillaries within normal brain. Arrows show capillaries in normal brain stained with  $\gamma$ -GTP and a tumor capillary without activity.  $\times 200$ .

to be decreased in some areas of normal brain surrounding these tumors.

To our knowledge, the observation that the normal BBB may have an enzymatic barrier to selectively resist increased permeability induced by biologically active compounds compared to systemic capillaries is unique. These findings add to our understanding of the biology of BBB in both normal and pathological conditions. The fact that intracarotid infusions of LTC<sub>4</sub> will selectively open the tumor barrier but not the BBB also has major clinical implications. Since intracarotid infusions of LTC<sub>4</sub> do not appear to produce any overt toxicity, this agent could be used to selectively increase delivery of a variety of antitumor moieties to tumor cells within the brain without increasing the exposure of normal tissue to the deleterious effects of antineoplastic agents.

### Acknowledgments

We thank Arthur W. Toga, Ph.D., for use of the Neuro-Imaging Laboratory and Ms. Maureen McCollough and Ms. Mayumi L. Smith for their technical assistance.

### References

1. Aharony D, Dobson P: Discriminative effect of gamma-glutamyl transpeptidase inhibitors on metabolism of leukotriene C<sub>4</sub> in peritoneal cells. *Life Sci* 35:2133-2142, 1984
2. Black KL, Betz AL, Ar DB: Leukotriene C<sub>4</sub> receptors in isolated brain capillaries. *Adv Prostaglandin Thromboxane Leukotriene Res* 17:303-311, 1987
3. Black KL, Hoff JT: Leukotrienes and blood-brain barrier permeability. *J Cereb Blood Flow Metab* 5 (Suppl): 263-264, 1985
4. Black KL, Hoff JT: Leukotrienes increase blood-brain barrier permeability following intraparenchymal injections in rats. *Ann Neurol* 18:349-351, 1985
5. Black KL, Hoff JT, McGillicuddy JE, et al: Increased leukotriene C<sub>4</sub> and vasogenic edema surrounding brain tumors in humans. *Ann Neurol* 19:592-595, 1986
6. Blasberg RG, Patlak CS, Jehle JW, et al: An autoradiographic technique to measure the permeability of normal and abnormal brain capillaries. *Neurology* 28:363, 1978 (Abstract)
7. DeBault LE:  $\gamma$ -Glutamyltranspeptidase induction mediated by glial foot process-to-endothelial contact in coculture. *Brain Res* 220:432-435, 1981
8. Fenstermacher JD, Cowles AL: Theoretic limitations of intracarotid infusions in brain tumor chemotherapy. *Cancer Treat Rep* 61:519-526, 1977
9. Groothuis DR, Fischer JR, Lapin G, et al: Permeability of different experimental brain tumor models to horseradish peroxidase. *J Neuropathol Exp Neurol* 41: 164-185, 1982
10. Kumagai AK, Eisenberg JB, Partridge WM: Absorptive-mediated endocytosis of osionized albumin and a  $\beta$ -endorphin-cationized albumin chimeric peptide by isolated brain capillaries. Model system of blood-brain barrier transport. *J Biol Chem* 262:15214-15219, 1987
11. Nakagawa H, Groothuis D, Blasberg RG: The effect of graded hypertonic intracarotid infusions on drug delivery to experimental RG-2 gliomas. *Neurology* 34: 1571-1581, 1984
12. Neuwelt EA, Barnett PA, Bigner DD, et al: Effects of adrenal cortical steroids and osmotic blood-brain barrier opening on methotrexate delivery to gliomas in the rodent: the factor of the blood-brain barrier. *Proc Natl Acad Sci USA* 79:4420-4423, 1982
13. Neuwelt EA, Barnett PA, McCormick CI, et al: Osmotic blood-brain barrier modification: monoclonal antibody, albumin, and methotrexate delivery to cerebrospinal fluid and brain. *Neurosurgery* 17:419-423, 1985
14. Neuwelt EA, Hill SA, Frenkel EP: Osmotic blood-brain barrier modification and combination chemotherapy: concurrent tumor regression in areas of barrier opening and progression in regions distant to barrier opening. *Neurosurgery* 15:362-366, 1984
15. Neuwelt EA, Rapoport SI: Modification of the blood-brain barrier in the chemotherapy of malignant brain tumors. *Fed Proc* 43:214-219, 1984



16. Partridge WM, Kumagai AK, Eisenberg JB: Chimeric peptides as a vehicle for peptide pharmaceutical delivery through the blood-brain barrier. *Biochem Biophys Res Commun* 146:307-313, 1987
17. Samuelsson B: Leukotrienes: mediators of immediate hypersensitivity reactions and inflammations. *Science* 220:568-575, 1983
18. Soter NA, Lewis RA, Corey EJ, et al: Local effects of synthetic leukotrienes (LTC<sub>4</sub>, LTD<sub>4</sub>, LTE<sub>4</sub>, and LTB<sub>4</sub>) in human skin. *J Invest Dermatol* 80:115-119, 1983
19. Toga AW, Santori EM, Samaja M: Regional distribution of flunitrazepam binding constants: visualizing K<sub>d</sub> and B<sub>max</sub> by digital image analysis. *J Neurosci* 6: 2747-2756, 1986
20. Tomiwa K, Hazama F, Mikawa H: Neurotoxicity of

K. L. Black, W. A. King, and K. Ikezaki

- vincristine after osmotic opening of the blood-brain barrier. *Neuropathol Appl Neurobiol* 9:345-354, 1983
21. Ueno A, Tanaka K, Katori M, et al: Species difference in increased vascular permeability by synthetic leukotriene C<sub>4</sub> and D<sub>4</sub>. *Prostaglandins* 21:637-648, 1981

Manuscript received August 30, 1989.

Accepted in final form January 16, 1990.

This work was supported by Grant 1R29 NS26523-01 from The National Institutes of Health and by grants from The Robert Wood Johnson Foundation and The Oxnard Foundation.

Address reprint requests to: Keith L. Black, M.D., Division of Neurosurgery, 74-140 CHS, UCLA Medical Center, Los Angeles, California 90024.